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Cytoprotective effects of sinapic acid on human keratinocytes (HaCaT) against ultraviolet B

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Abstract

Background: Sinapic acid is a phenolic compound in various plants, including spices, berries, citrus fruits, vegetables, grains, and oil seeds. It has been studied mainly for the various pathological symptoms it induces related to its anticancer, antioxidant, and neuroprotective effects. However, little has been studied about sinapic acid in human keratinocytes (HaCaT). Therefore, this study examined its reactive oxygen species (ROS) scavenging capacity, protective effect against DNA damage, and DNA repair mechanism.

Methods: To confirm the protective effects of sinapic acid on HaCaT irradiated by ultraviolet (UV) B, this study used the water-soluble tetrazolium salts (WST)-1 assay to determine the cytotoxicity of sinapic acid for verifying the cell survival rate and dichlorofluorescein diacetate (DCF-DA) to measure the changes in the concentration of ROS generated by UVB. This study also used the comet assay, cyclobutane pyrimidine dimer (CPD) formation, and malondialdehyde (MDA) assay to measure the degree of protection against cell damage. In addition, this study used quantitative real-time polymerase chain reaction (qRT-PCR) to measure the expression of repaired genes from cell damage.

Results: Sinapic acid protects cells from UVB-induced cytotoxicity and reduces ROS generation by UVB. This study showed that sinapic acid reduced the generation of tailed DNA, CPD, and lipid peroxidation, which are the indicators of DNA damage. Depending on the increase in the concentration of sinapic acid, the gene expressions of *XPC* and *ERCC1* (repair factors for damaged DNA) increased.

Conclusions: The findings of this study confirmed that sinapic acid is effective in removing ROS generated by UVB, reducing intracellular damage, and repairing damaged DNA in HaCaT. These results also showed that sinapic acid is effective in DNA damage repair, indicating its protective effect on HaCaT against UVB damage.

Keywords: Sinapic acid, HaCaT, ROS, DNA repair, Ultraviolet B

Background

Based on wavelength, ultraviolet (UV) rays are classified into UVC (200–290 nm), UVB (290–320 nm), and UVA (320–400 nm). With the highest energy level, UVC is mostly absorbed by the ozone layer in the atmosphere. With a shorter wavelength but higher energy level than UVA, UVB is a strong UV ray that causes sunburns and skin cancer (Kligman et al. 1985; Debacq-Chainiaux et al. 2005). Reactive oxygen species (ROS) are generated by UVB and are highly reactive such that they can act as intracellular signaling molecules at low concentrations but may damage cells by altering the molecular structure of DNA, lipids, and proteins at high concentrations (Evans et al. 1997).

UVB irradiation damages intracellular DNA through cleavage of single or double strands and photoproducts (Trosoko et al. 1965; Pardo et al. 2009). Nucleotide excision repair (NER) is an important damage repair system for such damages by UVB and the most commonly known repair system that can remove a wide range of DNA damages. NER is a complex process that requires about 20 or more proteins and compounds in human cells (Sugasawa et al. 1998). Its first step is to recognize the site of DNA damage through XPC complexed with HHR23B. XPC is

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the first to recognize DNA damage, and HHR23B enhances its activity (Sugasawa et al. 1998). The second step is to release the double strand of damaged DNA. As soon as the XPC–HHR23B complex recognizes the damaged region, TFIIH and XPG, which include helicases XPB and XPD, attach to the damaged site. XPB and RPA then stabilize open DNA and open the DNA damage sites of 30 bases (Schaeffer et al. 1994). Next, the actions of XPG and the ERCC1–XPF complex are required to remove the damaged DNA. XPG is cleaved from the 3′ to 5′ direction of the DNA damage, and the ERCC1–XPF complex is cleaved from the 5′ to 3′ direction. At the cleaved site, DNA polymerase resynthesizes and fills a new strand of complementary DNA for final repair (Benhamou and Sarasin 2000; Boer and Hoeijmakers 2000).

Sinapic acid is a phenolic compound found in various plants, including spices, berries, citrus fruits, vegetables, grains, and oil seeds (Sawa et al. 1999; Shahidi and Naczk 2004). Sinapic acid (4-hydroxy-3,5-dimethoxy cinnamic acid) is a derivative of cinnamic acid with a 3,5-dimethoxyl and 4-hydroxyl derivative in the phenyl group of cinnamic acid. Previous studies have reported various pathological symptoms associated with oxidative stress, inflammation, cancer, diabetes, neurodegeneration, and anxiety to have been induced by sinapic acid (Ansari et al. 2017; Balaji et al. 2014; Cherng et al. 2013; Zare et al. 2015; Yoon et al. 2007). This study examined the cytoprotective effects of sinapic acid on cell damage by UVB.

Methods

Cell culture

The human keratinocytes (HaCaT) used in this experiment were purchased from ATCC (USA). To incubate HaCaT cell strains, Dulbecco's modified Eagle's medium (Hyclone, USA) containing 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin (penicillin 100 IU/mL, streptomycin 100 μg/ml; Invitrogen, USA) was used. The cells were incubated at 37 °C with 5% CO_2.

Sample preparation

Sinapic acid was purchased from Sigma-Aldrich (USA) in purified (> 90%) powder, which was dissolved in dimethyl sulfoxide (Sigma-Aldrich) at an appropriate concentration for the experiment. HaCaT (1×10^6 cell/well) was incubated in a 6-well cell culture plate for 24 h, and sinapic acid was subsequently added at an appropriate concentration and preprocessed for six additional hours. A UV-B lamp (UVP, USA) was used to irradiate the cells with 40 mJ/cm^2 of UVB. The wavelength of UVB irradiation was measured with a fiberoptic Spectrometer System USB2000 (Ocean Optics, USA). To prevent dryness of cell, I opened the cell culture dish lid for UVB test after adding 1 mL of PBS to the cell culture dish and removed the PBS after the UVB test. After the PBS was removed, a new medium was added, followed by additional incubation of 24 h prior to use in the experiment.

Measurement of cell viability

The principles of the water-soluble tetrazolium salts (WST)-1 assay were used for the experiment on cell survival. Overall, 100 μl HaCaT (3×10^3 cells/well) was inoculated on a 96-well plate and incubated for 24 h. Afterward, sinapic acid was added at 10, 20, and 40 μM and 40 mJ/cm^2 and UVB was irradiated to these cells, which were subsequently incubated for additional 24 h. In addition, 10 μL of EZ-Cytox cell viability assay kit reagent (ITSBio, Korea) was added to the incubated cells for 1 h, and the absorbance was measured using a microplate reader (Bio-Rad, USA) at 490 nm. Cells in the control group were treated with same amount of DMSO, equal to the experimental group.

Quantitative analysis of intracellular ROS

In total, 10 μM of dichlorofluorescein diacetate (DCF-DA) was added as a dye to measure intracellular ROS. The cells were harvested after incubation for 30 min. And then, the change amount of ROS was measured. The fluorescence intensity was measured using a BD fluorescence-activated cell sorting caliber (FACSCalibur, flow cytometer, BD Biosciences, San Jose, CA, USA), at excitation and emission wavelengths of 485 and 535 nm, respectively. *N*-Acetyl-L-cysteine (NAC) was used as a positive control group that acts as a ROS scavenger.

Quantitative real-time PCR

Cultured cells were lysed using trizol reagent (Invitrogen, USA), and following which, 0.2 mL chloroform (Biopure, Canada) was added and left at room temperature. It was then centrifuged at 12,000 rpm at 4 °C for 20 min, thus separating the pellet containing proteins and the supernatant containing mRNA. A volume of 0.5 mL of isopropanol was added to the supernatant and left for 10 min at room temperature, then centrifuged at 12,000 rpm at 4 °C to precipitate the RNA. The RNA was washed with 75% ethanol and subsequently dried at room temperature after ethanol removal. Once dried, mRNA was dissolved in diethylpyrocarbonate (DEPC, Biopure) water for the experiment. The extracted RNA was quantified using a Nanodrop (Maestrogen, USA), and only RNA with a purity of 260 nm/280 nm (ratio 1:8) was used for the experiment.

In total, 10 μl of DNA was prepared by adding 1 μg RNA, 0.5 ng oligo dT18, and DEPC water in a PCR tube and processing it for 10 min at 70 °C to induce RNA denaturation. Subsequently, M-MLV reverse transcriptase (Enzynomics, Korea) was used to induce a reaction at 37 °C for 1 h to synthesize cDNA.

For qRT-PCR, 0.2 μM primers, 50 mM KCl, 20 mM Tris/HCl pH 8.4, 0.8 mM dNTP, 0.5 U Extaq DNA polymerase, 3 $MgCl_2$, and 1X SYBR green (Invitrogen) were mixed in a PCR tube to produce a reagent. PCR was performed with Linegene K (BioER, China) through 40 cycles (3 min per cycle) of denaturation, denaturation (94 °C, 30 s), annealing (58 °C, 30 s), and polymerization (72 °C, 30 s) at 94 °C. The significance of PCR was validated using a melting curve. The gene expressions were compared for analysis by normalizing the *β*-actin expression. The primers used for this experiment are shown in Table 1.

Comet assay (single-cell gel electrophoresis)

The Reagent Kit (Trevigen, USA) was used for the electrophoresis analysis. LMAgarose (37 °C) and dissolved 1×10^5 cells/ml were mixed in 1:10 (*v*/*v*) ratio, and 50 μl of this solution was added to the comet slide. The slide was placed in a refrigerator (4 °C) for 10 min and subsequently immersed in alkaline solution for 20 min at room temperature. After covering the alkaline electrophoresis solution, electrophoresis was performed at 21 V for 30 min. Then, the solution was removed using distilled water and immersed in 70% ethanol for 5 min. The agarose gel was dried at 37 °C for 15 min, placed in 100 μl SYBR Gold, and stained in a dark room for 30 min. After removing the remaining dye, the agarose gel was completely dried at 37 °C and observed through a confocal laser-scanning microscope (Olympus, Japan).

Cyclobutane pyrimidine dimer (CPD) formation

Genomic DNA was extracted from the cell sample, and 0.4 μg/ml genomic DNA was diluted through 1× assay. The heated reagent was rapidly cooled on ice for 15 min, and then, 50 μl of the sample solution was added to the ELISA plate coated with protamine sulfate and completely dried at 37 °C for 18 h. TDM-2 (CPD-specific monoclonal antibody clone) was incubated for 30 min in a well, washed with the PBS, and then the antibody was removed. The remaining TDM-2 antibody was treated with streptavidin-peroxidase and o-phenylenediamine (OPD) and measured at 492 nm using a microplate reader (Bio-Rad).

Malondialdehyde (MDA) assay (lipid peroxidation assay)

Using MDA lysis buffer, the cells were homogenized on ice and centrifuged (1300×*g*, 10 min) to separate 200 μl supernatant fluid. Thiobarbituric acid solution (600 μl) was added to each test tube and cooled at 95 °C for 1 h and then for 10 min on ice and dispensed into the 96 wells at 200 μl/well. A microplate reader (Bio-Rad) was used to measure at 586 nm.

Table 1 Lists of primers

Gene	Forward primer	Reverse primer
β-actin	GGATTCCTATGTGGGCGACGA	CGCTCGGTGAGGATCTTCATG
XPC	AGCAGCTTCCCACCTGTTC	GTGGGTGCCCCTCTAGTG
ERCC1	GGAGGCTGTTTGATGTCCTG	TTACACTGGGGGTTTCCTTG

Statistical analysis

Each experiment in this study was performed three times or more under the same conditions, and the results are expressed as mean and standard deviation. For each experiment, Student's *t* test was used to analyze all findings, with a *p* value of 0.05 or below considered as statistically significant.

Results

Cell survival analysis

Cell viability was measured via the WST-1 assay to determine the cytotoxicity of sinapic acid in HaCaT. In comparison with the 100% cell survival rate of the control group with no sinapic acid, the survival rates with sinapic acid at the concentrations of 5, 10, 20, 40, and 80 μM were 105, 121, 130, 136, and 97%, respectively. The survival rate did not decrease with up to 40 μM of sinapic acid but slightly decreased with 80 μM (Fig. 1a). In addition, the changes in cell viability were measured to examine the cytoprotective effect of sinapic acid in HaCaT damaged by UVB. In contrast to the 59% cell survival rate of the control group only irradiated by UVB, the groups preprocessed with 10 and 20 μM of sinapic acid showed the survival rates of 73 and 92%, respectively, indicating a concentration-dependent increase in cell survival by sinapic acid. Because the cell survival rate decreased to 79% with 40 μM, sinapic acid at the concentrations of 10 and 20 μM was used for further experiments (Fig. 1b).

Analysis of ROS scavenging capacity and protection capacity against DNA damage

In this experiment, the DCF-DA assay was used to confirm the ROS scavenging capacity of sinapic acid in HaCaT. In the control group, where HaCaT was irradiated with UVB without any sinapic acid, the ROS level increased to 6.9, but with 10 and 20 μM sinapic acid, it decreased to 3.6 and 2.2, respectively, depending on the concentration. This result verified the similar antioxidant effect with 1.5 of 10 mM NAC, which was used as a positive control group and a ROS scavenger (Fig. 2a). The comet assay was used to verify the cytoprotective effect of sinapic acid on DNA damage. As a result of the effect of sinapic acid on DNA damage in HaCaT treated by UVB, the DNA tail of the unprocessed control group of HaCaT increased from 4 to 53% after UVB radiation. However, after preprocessing with sinapic acid, it decreased to 37% with 10 μM of sinapic acid and 26% with 20 μM of sinapic acid depending on the concentration.

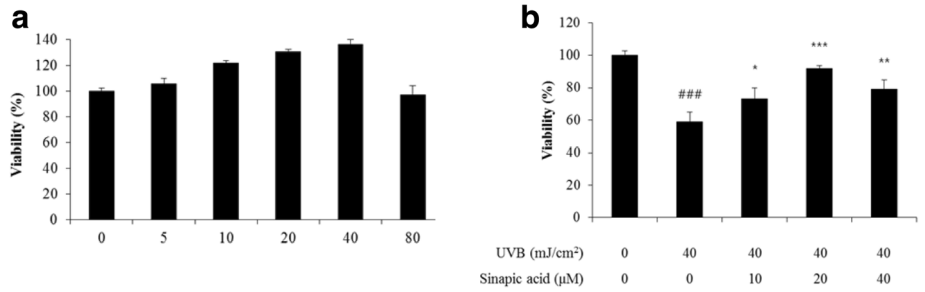

Fig. 1 Cell viability on sinapic acid in HaCaT. **a** Cell cytotoxicity of sinapic acid on HaCaT. No statistically significant cytotoxicity was observed in the groups treated with sinapic acid nontreated control group and sinapic acid. **b** The effect of sinapic acid on cell viability in UVB-irradiated HaCaT. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ###$p < 0.001$)

It was confirmed that sinapic acid protected HaCaT from UVB (Fig. 2b). As a typical photochemical reaction product of UV rays, CPD has damaged the DNA. To investigate the effect of sinapic acid on HaCaT damaged by UVB, the changes in CPD generation were observed. In contrast to level 1 in the control group (not processed with UVB), CPD generation increased to 100 after UVB irradiation. However, the generation decreased to 56 with 10 μM of sinapic acid and to 38 with 20 μM sinapic acid depending on the concentration. Thus, it was confirmed that sinapic acid protected cells from damage (Fig. 2c). MDA assay was performed to determine the degree of lipid oxidation of UVB-damaged cells. In comparison with 100% of the unprocessed control group, the CPD generation increased to 388% after only irradiated by UVB. However, it decreased to 245% with 10 μM of sinapic acid and to 121% with 20 μM. Therefore, it was verified that sinapic acid decreases lipid oxidation of cells in a concentration-dependent manner and increases its protective effect on HaCaT against UVB (Fig. 2d).

DNA repair capacity

DNA detects and repairs damage caused by ultraviolet radiation. With regard to the effects of sinapic acid on the expressions of *XPC* mRNA (DNA repair genes), the

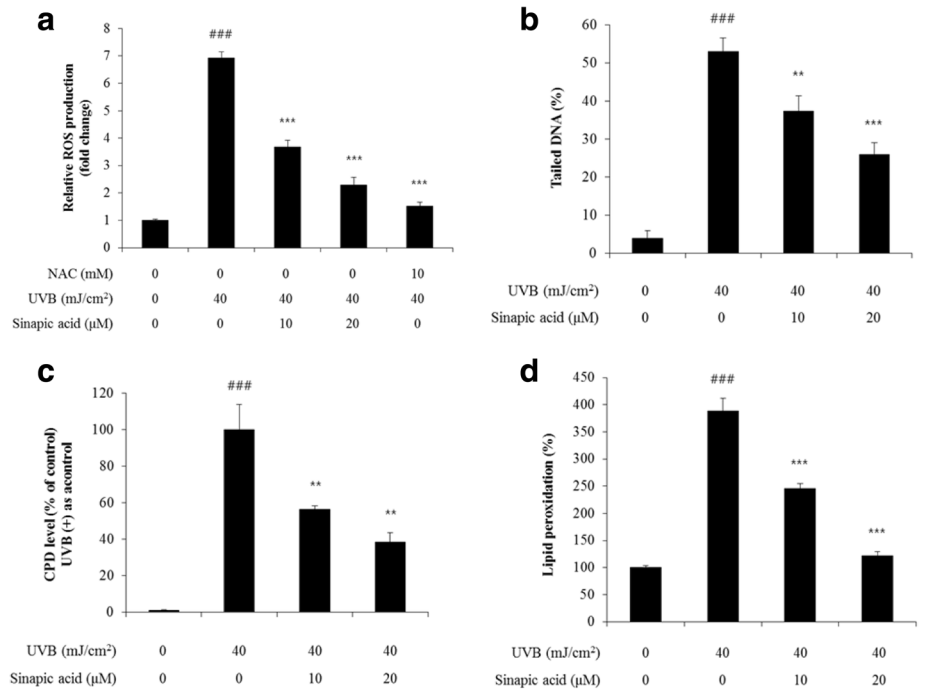

Fig. 2 Analysis of ROS removal capacity and DNA damage protection capacity of sinapic acid in HaCaT irradiated with UVB. **a** The ROS scavenging effect of sinapic acid in UVB-irradiated HaCaT. **b** The effect of sinapic acid on the amount of tailed DNA in HaCaT irradiated with UVB. **c** The effect of sinapic acid on the amount of CPD in HaCaT irradiated with UVB. **d** The effect of sinapic acid on the amount of lipid peroxidation in HaCaT irradiated with UVB.(**$p < 0.01$, ***$p < 0.001$, ###$p < 0.001$)

expression decreased to 0.29 after only irradiated by UVB but increased to 0.46 with 10 μM of sinapic acid and to 0.88 with 20 μM of sinapic acid depending on the concentration (Fig. 3a) in comparison with 1 in the unprocessed control group. ERCC1 is a gene that plays an important role in recognizing and eliminating DNA damage in the NER process, which is a DNA damage repair mechanism. The expression of *ERCC1* mRNA in HaCaT decreased to 0.40 after UVB irradiation in comparison with 1 in the unprocessed control group with no UVB irradiation. However, the expression increased to 0.78 with 10 μM of sinapic acid and to 1.17 with 20 μM depending on the concentration in comparison with 1 in the unprocessed control group (Fig. 3b). Therefore, it is verified that sinapic acid protects HaCaT damage by increasing the expressions of *XPC* and *ERCC1* mRNA, which are DNA repair factors of UVB-damaged DNA.

Discussion

ROS are highly chemically reactive in an unbound and unstable state that includes oxygen atoms. This type of reactivity causes other materials to be oxidized and damaged (Beckman and Ames 1998). Normally, ROS are at equilibrium with the corresponding antioxidants. However, excessive generation of oxidants owing to increased oxygen in the body or imbalance by decreased antioxidants causes oxidative stress. This type of stress intervenes to cause not only aging but also diseases because it damages and deforms DNA, lipids, and protein as well as cause cell necrosis or apoptosis (Sagara et al. 1998). In this study, NAC, which is used as an antioxidant standard substance, was further tested using DCF-DA to confirm the efficacy of sinapic acid to remove ROS that damages these cells. As a result, it was confirmed that sinapic acid has an antioxidant effect similar to NAC in Fig. 2a.

DNA damage at the cellular level is associated with various genetic diseases, cancer, and aging. CPD and pyrimidine (6–4) pyrimidone (6–4 PP) are phytochemical reaction products induced by UVB irradiation that causes DNA damage and mutations that may lead to skin cancer (Pfeifer and Besaratinia 2012). Fortunately, the human body activates the repair system NER to recover from this damage. An emphasis is placed on this system because when it fails to accurately operate due to mutation by defective factors, various diseases may be induced (Boer and Hoeijmakers 2000). In this study, the CPD ELISA kit was used to verify the effect of sinapic acid on CPD, a typical photochemical reaction product induced by UVB irradiation. The concentration of CPD, which increased rapidly after UVB irradiation, decreased when treated with sinapic acid depending on the concentration (Fig. 2c). Moreover, the amount of tailed DNA, a damaged fragment of DNA owing to UVB, also decreased when treated with sinapic acid depending on the concentration (Fig. 2b). ROS-induced oxidative stress oxidizes DNA and proteins, leading to structural changes in them, and oxidizes fatty acids of the cell membrane to increase MDA, a lipid peroxide. This increase in MDA leads to oxidative damage of cells, thereby decreasing cell function and causing various chronic diseases, such as cancer (Gloire et al. 2006). In this study, the final product of lipid oxidation was measured using the MDA assay. When treated with UVB, lipid peroxidation increased to 388% but decreased when treated with sinapic acid depending on the concentration (Fig. 2d). These results suggest that sinapic acid protects cells from DNA damage caused by UVB.

To maintain homeostasis, cells have DNA repair mechanism with multiple processes to stop cell cycle or restore genetic stability. Despite this mechanism, apoptosis occurs if the repair is not accurately performed. Therefore, cell cycle arrest and DNA repair are very important defense mechanisms against DNA damage (Sancar et al. 2004). qRT-PCR was performed to confirm the effects of sinapic acid on DNA protection at the genetic level, and changes were observed in the gene expression related to DNA damage. XPC is the first to recognize damaged DNA in the NER process and allows

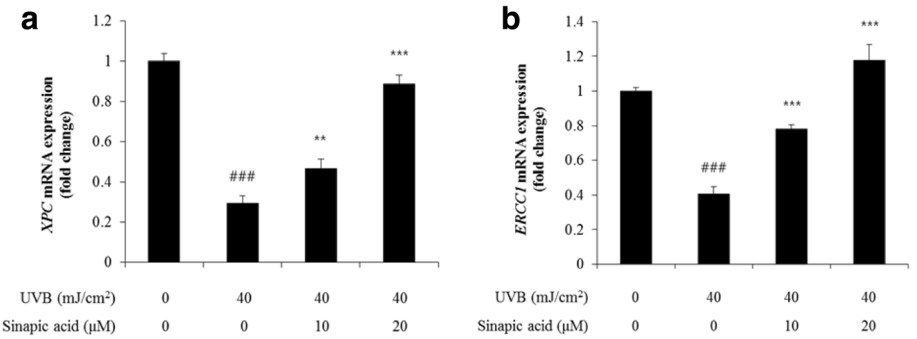

Fig. 3 Analysis of DNA damage repair capacity of sinapic acid in UVB-irradiated HaCaT. **a** The effect of sinapic acid on expression of *XPC* mRNA in UVB-irradiated HaCaT. **b** The effect of sinapic acid on expression of *ERCC1* mRNA in UVB-irradiated HaCaT. (**$p < 0.01$, ***$p < 0.001$, ###$p < 0.001$)

other NER factors to be collected (Sugasawa et al. 1998). ERCC1 recognizes DNA damage, removes the damaged sites, and indicates cellular NER activity as the most important gene for the recombination of homologous chromosomes (Reed 1998; Gaillard and Wood 2001). The expressions of *XPC* and *ERCC1* mRNA, which play an important role in the NER process activated for DNA repair, decreased when irradiated by UVB but increased when treated with sinapic acid depending on the concentration (Fig. 3a, b). Therefore, it is suggested that sinapic acid protects cells from DNA damage and intervenes to repair the damage as its cell-protective effects.

Conclusions

This study verified the protective effects of sinapic acid from DNA damage by observing the changes in tailed DNA, CPD, and lipid peroxidation as the indicators of DNA damage. HaCaT cells were treated with sinapic acid at various concentrations and irradiated with UVB to induce cellular damage, after which the changes were observed. In addition, this study examined the expression of *ERCC1*, which directly removes the damaged DNA regions, and *XPC* that is the first to recognize DNA damage among the genes in which sinapic acid repairs intracellular DNA. As a result, it was verified that sinapic acid protects HaCaT against cellular damage induced by UVB. Sinapic acid eliminated RO and reduced the generation of tailed DNA, CPD, and lipid peroxidation in a concentration-dependent manner to increase the expression levels of *XPC* and *ERCC1*, which are important in the repair process of damaged cells. These results suggest that sinapic acid reduces UVA-induced DNA damage in HaCaT and is thus effective in protecting cells from damage.

Abbreviations

CPD: Cyclobutane pyrimidine dimer; DMSO: Dimethyl sulfoxide; ERCC1: ERCC excision repair 1, endonuclease non-catalytic subunit; FACS: Fluorescence-activated cell sorting; FBS: Fetal bovine serum; HaCaT: Human keratinocyte; HHR23B: RAD23 homolog B, nucleotide excision repair protein; LMAgarose: Low melting agarose; MDA: Malondialdehyde; NAC: *N*-Acetyl-L-cysteine; NER: Nucleotide excision repair; PBS: Phosphate-buffered saline; qRT-PCR: Quantitative real-time polymerase chain reaction; ROS: Reactive oxygen species; RPA: Replication protein A; TFIIH: Transcription factor II human; UV: Ultraviolet; WST: Water-soluble tetrazolium salts; XPB: ERCC excision repair 3, TFIIH core complex helicase subunit; XPC: XPC complex subunit, DNA damage recognition and repair factor; XPD: ERCC excision repair 2, TFIIH core complex helicase subunit; XPF: ERCC excision repair 4; XPG: ERCC excision repair 5, endonuclease

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Availability of data and materials

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Authors' contributions

KH did all of the research background such as the experiments, data collection, and statistical analysis as well as manuscript drafting. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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